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Study on the effect of citric acid adaptation toward the subsequent survival of *Lactobacillus plantarum* NCIMB 8826 in low pH fruit juices during refrigerated storage

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Abstract

Pre-treatment of stationary phase cells of *Lactobacillus plantarum* NCMIB 8826 with citric acid (pH 3 to 6) for a short period of time significantly improved subsequent cell survival in several highly acidic fruit juices namely cranberry (pH 2.7), pomegranate (pH 3.5), and lemon & lime juices (pH 2.8). Although the mechanism for this adaptation is still unclear, the analysis of the cellular fatty acid content of acid adapted cells and the reverse transcription polymerase chain reaction (RT-PCR) showed a significant increase (by ~1.7 fold) of the cellular cyclopropane fatty acid, cis-11,12-methylene octadecanoic acid ($C_{19:0cyclow7c}$) and a significant upregulation (~12 fold) of cyclopropane synthase (*cfa*) were observed, respectively, during acid adaptation. It is likely that these changes led to a decrease in membrane fluidity and to lower membrane permeability, which prevents the cells from proton influx during storage in these low pH fruit juices.

Keywords: Acid adaptation, probiotics, cranberry, lemon, pomegranate, stress adaptation, cis-11,12-methylene octadecanoic acid, cyclopropane fatty acid, RT-PCR, cyclopropane synthase (*cfa*), and β-ketoacyl-acyl carrier protein synthase III (*FabH*)

1 Introduction

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill *et al*., 2014). The consumption of probiotics has demonstrated several positive effects on human health (de Almada *et al*., 2015) resulting in the development of appropriate methods and technologies for the incorporation of probiotics into various food products. Although probiotics have been mainly incorporated into yoghurt (Batista *et al*., 2017), cheeses (Silva *et al*., 2018), and ice cream (Balthazar *et al*., 2018), a challenging task for the food industry is to expand their use in a wider range of non-dairy probiotic products such as fruit juices (Kandylis *et al*., 2016; Panghal *et al*., 2017; Shori, 2016).

Fruit juices is a promising vehicle for the delivery of probiotics as they contain relatively high concentrations of sugars, essential vitamins and minerals, which could be used as energy and nutrient sources for the survival of probiotics during storage (Ding and Shah, 2008) and offer an alternative choice to consumers with lactose intolerance (Prado *et al*., 2008). Moreover, the fruit juice market is big, as fruit juices are easily consumed by people from various ages. Many fruit juices are acidic (pH 2.5 - 3.8) which is harmful to most bacteria (Champagne *et al*., 2005; Sheehan *et al*., 2007). This is a major issue for developing fruit juices as a new probiotic delivery vehicle. However, a large number of cells die during the production process and storage due to the low pH conditions (Gueimonde *et al*., 2004; Shah, 2000; Vinderola *et al*., 2000). The minimum dose required for their efficacy in order to convey health benefits is suggested to be approximately 10^7 to 10^9 CFU/g at the time of consumption (Corcoran *et al*., 2007; Rivera-Espinoza and Gallardo-Navarro, 2010). Therefore, many approaches such as encapsulation (Nualkaekul *et al*., 2012) and induction of

acid tolerance response (Saarela *et al*., 2011) have been used to enhance the survival of the bacteria in acidic fruit juices.

A short exposure of probiotic bacteria to acid stress prior to their incorporation into fruit juices followed by subsequent storage at refrigerated temperatures (Saarela *et al*., 2011) is a viable approach to reduce cell injury and death during processing, storage, and passage through the gastrointestinal tract. Additionally, this approach would require less process alterations, capital investment and product re-design compared to other ones. This strategy would allow cells to activate defence mechanisms against acidic conditions, such as maintenance of ΔpH homeostatic, alteration of cell envelope, as well as protection and restoration of proteins and DNA (Lorca and de Valdez, 2009; Spano and Massa, 2006; Van de Guchte *et al*., 2002). This behaviour has been previously reported in many lactic acid bacteria (LAB). For example, exposure of *Lactobacillus acidophilus* for 15 to 60 min to acidified MRS at different pH values (pH 3.8 to 6.0) resulted in an increase of acid tolerance during subsequent incubation in acidified MRS (pH 3) (Lorca *et al*., 1998). Similarly, exposure of *Bifidobacterium longum* to acidified MRS (pH 4 – 5) supplemented with 0.05% L-cysteine for 16 h resulted in increased acid resistance and consequently to increased survival in artificial gastric solution (pH 2) (Sanchez *et al*., 2007). Furthermore, exposure of *Lactobacillus casei* in acidified MRS (pH 4.5) for 1 h was able to enhance its survival in acidified MRS (pH 3.5) compared to 1 h exposure in acidified MRS (pH 5 and 6) (Wu, He, *et al*., 2014). Based on this concept, several studies have been conducted assessing various acids for the adaptation stage, such as lactic acid (Lorca and de Valdez, 2001), hydrochloric acid (Rallu *et al*., 1996) and malic acid (Saarela *et al*., 2011). Interestingly, García-Quintáns *et al*. (1998) highlighted the positive effects of citrate in enhancing the acid tolerance of *Lactococcus lactis*. The investigation of citrate is important as the latter is naturally present in high levels especially in citrus fruit juices or added as a preservative in a wide variety of

fruit juices. Although the exact role of citric acid in acid tolerance of *Lactobacillus* species is not well understood, it is believed that increasing the degree of saturation and the level of cyclopropane formation could potentially improve their survival (Montanari *et al*., 2010). Moreover, two studies have shown the overexpression of the cyclopropane synthase (*cfa*) gene in *L. plantarum* (Seme *et al*., 2015) and of the β-ketoacyl-acyl carrier protein synthase III (*fabH*) gene in *L. bulgaricus* (Fernandez *et al*., 2008) during acid stress.

Cyclopropane synthase is an enzyme which catalyzes the conversion of the cis-double bond of unsaturated fatty acids to the cyclic ring of their cyclopropane derivatives. The expression of *cfa* is generally under control of s^{70}-dependent promoter and s^{S}-dependent promoter (Eichel *et al*., 1999). However, during acidic condition the s^{S} protein competes with the s^{70} protein for binding with the RNA polymerase at the promoter sites resulting in the activation of *cfa* transcription in *L. lactis* (Budin-Verneuil *et al*., 2005). In contrast with the β-ketoacyl-ACP synthase III is an enzyme which forms the first β‑ketoacyl-ACP intermediate in the first step of the elongation process of fatty acid. The expression of *fabH* gene is under control by *fabH* promoter located in the *rpmF-plsX-fab* operon (Podkovyrov and Larson, 1996). My *et al*. (2013) also indicated that the activation of *fabH* not only is involved in the upregulation of *fadR* but also the absence of the nucleotide guanosine 3',5'-bisdiphosphate (ppGpp) in *Escherichia coli*. Additionally, The *fabH* promoter was subject to *relA* which is responsible for the synthesis of ppGpp during the stringent response to the amino acid starvation in *E. coli* (Podkovyrov and Larson, 1996).

The aim of the present study was to evaluate the potential use of acid adaptation, particularly in citric acid which is present in a wide variety of juices, to enhance the survival of a model potential probiotic strain (*L. plantarum* NCIMB 8826) during storage in acidic

fruit juices and investigate the upregulation of *cfa* and *fabH* genes which could be possible involved in acid tolerance of this bacterium.

2 Material and methods

2.1 Bacterial strains

L. plantarum NCIMB 8826 (National Collection of Industrial and Marine Bacteria, UK), isolated from human saliva, was used throughout this study. The stock culture was stored at -80 °C in de Man, Rogosa and Sharpe medium (MRS, Oxoid, UK) containing 10% (v/v) glycerol (Sigma-Aldrich, UK).

2.2 Fruit juices

Three commercial fruit juices, namely cranberry (Oceanspray™), pomegranate (Pureplus™) and lemon & lime (This water®), available in the UK market, were used in this study. Commercial juices were preferred as they are normal juices which contain additional compounds such as preservatives (e.g. citric acid), acids, colouring and flavouring agents. Therefore, if the microorganism is able to perform well in this juice it would be able to also perform well in normal juices. The cranberry juice comprised water (83%), cranberry juice from concentrate (27%), sugar (11 g/100 ml), and vitamin C (32 mg/100 ml). The pomegranate juice comprised water (68%), pomegranate from concentrate (32%), sugar (11.7 g/100 ml), protein (0.2 g/100 ml) and fat (0.2 g/100 ml). The lemon & lime juice comprised fresh lemon and lime (100%). Sterilization of fruit juices by filtrating through 0.2 μm membrane was performed before use. All fruit juices used in this experiment contained no preservatives or additives.

2.3 Early stationary phase cells

A two-step propagation procedure was used to prepare stationary phase cells. The cells from a cryovial at -20°C were thawed on ice and transferred onto MRS agar (Oxoid, UK) by streak plating. After incubation for 3 days at 37 °C, a single colony was obtained and inoculated into a glass tube containing 10 ml of MRS (Oxoid, UK). Following an overnight incubation for 18 h at 37°C, bacterial growth was measured using a spectrophotometer (Biomate 3, Thermo Scientific, UK) at 600 nm. An appropriate volume of this overnight culture was inoculated as such that the starting OD_{600} of the MRS culture was approximately 0.2. The culture was then incubated at 37 °C in an orbital shaker (KS 501 digital, IKA, Germany) set at 200 rpm. The growth *L. plantarum* NCIMB 8826 was monitored every 3 h, for 30 h in order to identify the early stationary phase of the cell growth and thus the optimum harvest time for the cell adaptation experiments.

2.4 Acid adaptation

Fifty ml of early stationary phase cell culture (~10^{10} CFU/ml; ~15 h culture) was harvested by centrifugation (Heraeus™ Multifuge™ X 3 Centrifuge, Thermo Scientific, UK) at 3,500 g for 15 min. The pellet was washed twice with 0.1 M phosphate buffer saline (PBS, Oxoid, UK) at pH 7.3 and resuspended in the same diluent (5 ml of PBS). A total volume of 250 µl of this cell suspension was then transferred into fresh acidified MRS media (pH 2 to 6, 25 ml), while unmodified MRS (pH 6.4, 25 ml) was used as a control. The pH of the acidified MRS was adjusted using 99.5% citric acid powder. Citrate was used in our experiments as it is one of the most common acids present in juices either naturally (in even higher concentrations than those used here) or as a preservative. However, the pH 2 was excluded from this experimental set as MRS became cloudy when it was acidified to pH 2.

These media were filter-sterilised before use with syringe filters (0.2 μm pore size; Minisart, Sartorius AG, Germany). The cells suspended in these media were then incubated at 25 °C for 1 h in order to avoid cell growth during acid adaptation. After incubation, the cells were collected from each acidified solution by centrifugation at 3,500 g for 15 min. The pellets were washed twice in 0.1 M PBS (pH 7.3), followed by centrifugation at 3,500 g for 15 min and finally resuspended in 2.5 ml of each of the fruit juices. Then, 250 μl of this cell suspension was used to inoculate 25 ml of each of the corresponding fruit juices which were previously filter-sterilised contained in 50 ml sterile plastic containers. Consequently, the initial cell concentration in the fruit juices was approximately ~10^8 CFU/ml while they were stored at 4 °C.

2.5 Bacterial enumeration and statistical analysis

The viable cell counts were determined by spread plate method immediately after inoculation of all juices and after 1, 2, and 3 days of storage for cranberry juice, and every week for up to 6 weeks for pomegranate and lemon & lime juices. The plates were incubated anaerobically in an anaerobic chamber at 37°C for 3 days prior to counting using a colony counter (Colony counter SC5, Stuat™, UK). The results were expressed as colony forming units per ml (CFU/ml). The data shown are expressed throughout as mean ± SD of three independent replicate survival experiments. The effects of the acid adaptation on the survival of *L. plantarum* in cranberry juice were analysed using 1-way ANOVA and Tukey's post-hoc tests, while those in pomegranate and lemon & lime juices were analysed using a paired t-test.

2.6 Determination of cellular fatty acid composition

Fifty ml of early stationary phase cells were prepared as mentioned above, washed twice with 0.1 M PBS (pH 7.3) and collected by centrifugation at 3,500 g for 15 min. The pellets were then transferred in parallel in 50 ml acidified MRS (pH 3) and a similar volume unmodified MRS (pH 6.4) and incubated for 1 h at 25 °C. Following incubation, the cells were harvested by centrifugation at 3,500 g for 15 min, washed twice with 0.1 M PBS (pH 7.3, 50 ml), and frozen at -80 ^{o}C overnight before freeze drying (VirTis BenchTop™ K Series, SP Scientific, UK). The fatty acids were extracted from 20 mg of each freeze-dried sample according to a standard protocol for fatty acid analysis (Miller, 1982, Kuykendall *et al*., 1988). The mixtures of fatty acid methyl esters produced were analysed using a Hewlett-Packard 5898A microbial identification system (Microbial ID Inc, USA) equipped with a gas chromatography system (Hewlett-Packard model 5980), a flame ionization detector (Hewlett-Packard models 3392), and an autosampler (Hewlett-Packard models 7673). The detection was performed using a 25 m x 0.2 mm 5% phenylmethyl silicone capillary column. All peaks were automatically integrated and the percentages of each fatty acid were calculated.

2.7 Gene expression analysis by reverse transcription-polymerase chain reaction (RT-PCR)

The transcription of cyclopropane synthase (*cfa*) and *β*-ketoacyl-acyl carrier protein synthase III (*FabH*) were evaluated in this study. The 16S rRNA gene was used as the reference gene because it is one of the housekeeping genes exhibiting constant RNA transcription in *L. plantarum* NCIMB 8826 cells independently of the environmental conditions (Lee *et al*., 2008).

2.7.1 Design of primers for RT-PCR

Primer-BLAST, a free primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), was used to design the specific primers for the targeted genes. After obtaining the results, each pair of primers was checked for their specificity toward the genome of *L. plantarum* WCFS1 using NCBI Blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Furthermore, the primer dimers and hairpin structures were calculated by OligoEvaluator (http://www.oligoevaluator.com/Login.jsp) before the PCR experiments were performed.

2.7.2 Preparation of samples for RNA extraction

The pellets obtained from 50 ml of early stationary phase cells prepared as mentioned above were collected by centrifugation at 3,500 g for 15 min and washed twice with 0.1 M PBS (pH 7.3, 50 ml). They were resuspended and incubated for 1 h at 25 °C in 50 ml of acidified MRS (pH 3) and an equal volume of unmodified MRS (pH 6.4). After incubation, both cell samples were collected by centrifugation at 3,500 g for 15 min and then kept in 25 ml of RNA stabilization reagent (RNA*later*, Qiagen, UK) before use. The pre-treatment cells were collected from the reagent solutions by centrifugation at 3,500 g for 15 min and resuspended in 2.5 ml nuclease free water (Qiagen, UK). One ml of each cell suspension was transferred into a 2 ml microtube containing acid-washed 106-mm-diameter glass beads. The samples were disrupted thrice using a Mini-Beadbeater (Biospec, UK) for 1 min each time with 1 min intervals when they were cooled on ice. After mechanical cell lysis, the aqueous phase was collected by centrifugation for 10 min at 15,000 g (4 °C) and transferred into a new sterile 1.5 ml microtube. The RNA of each sample was isolated using RNeasy Mini Kit (Qiagen, UK) according to the manufacturer's instructions. All RNA preparations were treated with RNase-free DNase for 20 min at 37 °C during mRNA extraction. Finally, the

RNA was dissolved in 20 µl of nuclease-free water and stored at -20 °C until use. The concentration and purity of each RNA sample were determined using a NanoDrop ND-1000 (Thermo Scientific, UK).

2.7.3 RT-PCR quantification

The genes of interest including the reference gene (Table 1) were amplified using real time RT-PCR with QuantiFast SYBR Green RT-PCR Kit (Qiagen, UK). The amplification was run in triplicate for each sample in white 96 well plates (Roche Applied Science) using the LightCycler® 480 RT-PCR system. Before the amplicons were analysed, preliminary RT-PCR experiments were conducted by preparing 10-fold serial dilutions of the extracted mRNA to obtain the E value for each set of primers and identify which dilution should be used. Reactions were carried out in a total volume of 25 µl, containing 2 µl of mRNA, 1 µl of each primer (0.01 nM), 12.5 µl of 2X QuantiFast SYBR Green RT-PCR Master Mix, 0.25 µl of QuantiFast RT Mix and 8.25 µl of RNase free water. An aliquot of 2 µl of RNase free water was included as a non-template in each run to identify possible mRNA contamination. The RT-PCR cycler conditions were set as follows: Reverse transcription at 50 °C for 10 min, PCR initial activation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 10 sec, annealing at 60 °C for 30 sec, and elongation with measurement of fluorescence at 72 °C for 30 sec. The cycles were followed by a melting curve analysis at 95 °C for 1 min, 55 °C for 30 sec, and a slow increase with a heating rate of 0.1 °C per sec to 95 °C with continuous fluorescence acquisition. The relative expression ratio of each candidate gene was determined by comparison with the expression levels of the reference gene. The RT-PCR efficiency (E) for each pair of primers was calculated by using serial 1:10 dilutions of starting mRNA template for 5 times with RNase free water.

3 Results and Discussion

3.1 Survival of acid-adapted cells in three fruit juices

The exposure in acidified MRS (pH 3 to 6) for 1 h had no detrimental effects on survival as initial viable cell numbers (0 h) were similar to the control in all fruit juices (unmodified MRS, pH 6.4). Furthermore, increasing exposure time did not enhance subsequent cell survival; moreover, incubation longer than 3 h, reduced the numbers of viable cells (data not shown).

In cranberry juice (Figure 1), adapted cells in acidified MRS at pH values ≥3 significantly improved subsequent cell survival (24 to 48 h) compared to the control cells in unmodified MRS at pH 6.4. The greatest survival was obtained after adapting cells in acidified MRS to pH 3, followed by pH 4, 5, and 6. The adapted cells in acidified MRS pH 3 and 4 were able to survive in cranberry juice for 72 h, at low concentrations (~10^3 and ~10^2 CFU/ml, respectively). In pomegranate and lemon & lime juices (Figure 2), the survival of *L. plantarum* NCIMB 8826 was higher than in cranberry juice. The adapted cells in acidified MRS (pH 3) exhibited an 1 log CFU/ml higher survival for the 1st and 2nd week compared to the control cells in both juices. However, no significant differences were observed for the 3rd week and beyond. These results indicate that acid adaptation had a positive effect on survival in pomegranate and lemon & lime juices but only for the initial stages of storage, suggesting that the effect was not permanent.

The significant loss in cell viability of *L. plantarum* NCIMB 8826 during storage in these fruit juices was most likely due to the low pH of the juices which ranged between 2.5 and 3.8 and is it known to have an adverse effect on bacterial survival (Champagne *et al*., 2005; Sheehan *et al*., 2007). More specifically, the pH of cranberry, pomegranate and lemon

& lime juices were 2.7, 3.5, and 2.8, respectively. Interestingly, although the pH values of cranberry and lemon & lime juice were very similar, the survival rates were considerably different as cells died within the first week of storage in cranberry juice whereas in lemon & lime juice they were viable until week 5. This could be due to the presence of high levels of phenolic compounds in this specific cranberry juice (Nualkaekul and Charalampopoulos, 2011) and promegranate juice (Gil *et al*, 2000) which has have been reported previously. Certain phenolic compounds are known to have strong antimicrobial effects similar to those of benzoic (Sheehan *et al*., 2007) and coumaric acid (Landete *et al*., 2007). Moreover, the presence of phenolic compounds in fruit juices used in this study might also explain why cells died quicker in pomegranate juice compared to lemon & lime juice (4 weeks versus 5 weeks) despite the fact that the pH in the former was higher. Furthermore, these results could be explained by the fact that cells were pre-adapted to citric acid which is the main antimicrobial in the lemon & lime juice, resulting in higher survival to the latter juice compared to the others.

The effect of pH towards survival of the cells has been also observed by Seme *et al*. (2015), although a different strain, *L. plantarum* KR6, and a different experimental set up were used. Their work demonstrated that non-acid stressed cells of *L. plantarum* KR6 exposed to acidified MRS (pH 4.5 using HCl) for 30 min, increased survival in acidified MRS (pH 2) by about 100 times compared to the control (cells exposed to MRS at pH 7 for 30 min). The researchers suggested that this behaviour might relate to the upregulation of genes involved in membrane fatty acid biosynthesis, particularly *cfa*, as determined by qPCR analysis (Seme *et al*., 2015). To this end, the higher survival rates of *L. plantarum* NCIMB 8826 in cranberry juice (24 to 48 h) after further acid adaptation (pH 6, 5, 4 and 3) might be associated with changes in the composition of the membrane fatty acids which is used as a

mechanism by the cells in order to regulate the proton permeability through their cell membrane (Lemos and Burne, 2008; Zhang and Rock, 2008).

Moreover, this phenomenon could be explained on the basis of the study of Pieterse *et al*. (2005) who investigated the effect of the dissociated and undissociated forms of lactic acid and that of hydrogen ions alone on *L. plantarum* WCFS1. The levels of gene/operon expression were measured during continuous steady state experiments, in which the pH was controlled by automatic titration with 10 M sodium hydroxide. The degree of undissociated/dissociated forms was varied by adding 300 mM of sodium lactate at two different pH values, at pH 6.0 (lactate effect; dissociated>undissociated form) and pH 4.8 (undissociated lactic acid effect; undissociated>dissociated form). Gene expression analysis indicated that during acid stress a small number of genes/operons were differentially expressed in response to the hydrogen ions and the dissociated form of lactic acid. On the other hand, stressing the cells with the undissociated form of lactic acid resulted in the overexpression of several other genes/operons in *L. plantarum* WCFS1 (Pieterse *et al*., 2005). This suggests that the undissociated form of the acid also has an effect on the cell independently of its activity through the release of protons as an acid.

3.2 Alteration of the cell membrane fatty acid composition

The total amount of saturated fatty acids increased by ~2.3% (cyclopropane is not included in the calculations) after acid adaptation whereas the unsaturated fatty acids decreased by ~1.3% (Table 2). However, these changes were not statistically significant leading to the conclusion that the degree of saturation/unsaturation of membrane fatty acids did not change during acid adaptation and therefore did not influence the survival of *L. plantarum* NCIMB 8826 during storage in fruit juices. The major fatty acids of *L. plantarum*

NCIMB 8826 were myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1w7c}$), oleic acid ($C_{18:1w9c}$), cis-vaccenic acid ($C_{18:1w7c}$) and nonadecenoic acid ($C_{19:1}$). These acids constituted about 90% of the total fatty acids content, which is similar to what has been previously reported for *L. plantarum* 2004, with the main exception being the presence of nonadecenoic acid ($C_{19:1}$) in *L. plantarum* 2004 (Johnsson *et al*., 1995)

The role of membrane fatty acid composition in the acid stress response of LABs has been the subject of a number of studies; however, the results have been contradictory. The general hypothesis is that by modifying their membrane fatty acid composition, the cells are able to adjust their membrane viscosity to respond to environmental stimuli (Guerzoni *et al*., 2001; Montanari *et al*., 2010; Streit *et al*., 2008; Wu *et al*., 2012). More specifically, in the case of acid stress, the cells reduce their membrane fluidity to protect the cytoplasm from the influx of hydrogen protons by either increasing the degree of saturation or decreasing the degree of unsaturation of the membrane fatty acids. Saturated fatty acids are linear and tightly packed together resulting in the production of a bilayer which has a high phase transition and low permeability, while the cis-double bond unsaturated fatty acids introduces a twist in the chain, which disrupts the order of the bilayer and leads to lower transition temperatures and higher permeability (Zhang and Rock, 2008). However, as mentioned above, we did not observe these changes in the membrane fatty acid composition, although a trend was observed suggesting an increase in the total saturated and a decrease in the total unsaturated fatty acids. The discrepancies observed in the published work could be attributed to differences in the intrinsic properties of the strains used, low accuracy and reproducibility in fatty acid analysis, as well as considerable differences in the experimental design. For example, some studies carried out acid stress experiments under growth conditions (at low pH or non-controlled pH), whereas others under no-growth conditions in buffer solutions or media.

Interestingly, the fatty acid analysis indicates that the relative concentration of the cyclopropane fatty acid, cis-11,12-methylene octadecanoic acid ($C_{19:0cyclow7c}$) which was generally known as lactobacillic acid, significantly increased (from ~ 6% to ~ 10%) in the acid adapted cells compared to the control cells (Table 2). Although there are two cyclopropane fatty acids, dehydrosterculic acid (cis-9,10-methylene octadecanoic acid, $C_{19cyclow9c}$) and lactobacillic acid, which are normally found at high percentages in LAB after acid stress and cold stress (Montanari *et al*., 2010), only lactobacillic acid was detected in this study. According to Broadbent *et al*. (2010) and Wu, Huang, *et al*. (2014), cyclopropane fatty acids are very important in controlling the biophysical properties of the cell membrane. Additionally, an increase in the amount of cyclopropane (octadecanoic acid, $C_{19cyclo}$) due to acid stress was observed previously in *L. casei* strain Zhang (Wu *et al*., 2012). Considering the results of this study in conjunction with the previous work with lactobacilli, it can be assumed that a possible increase in cyclopropane fatty acid in *L. plantarum* NCIMB 8826 improved cell survival during storage in the fruit juices by lowering the cell membrane fluidity and consequently the proton influx. Moreover, an increase in the cyclopropane content of *L. plantarum* DSM 10492 during growth at pH 5.5 in the presence of some phenolic compounds, such as caffeic acid, ferulic acid and tannin in the growth medium was observed in a previous study (Rozes and Peres, 1998). It is possible that the increased levels of cyclopropane in the cell membrane not only reduce proton influx but also protect the cell from phenolic compounds present in the fruit juices.

3.3 Gene expression analysis by RT-PCR

As the amount of cyclopropane and saturated fatty acids were increased due to acid adaptation, the *cfa*; a gene which involves in catalysing the conversion of the cis-double bond of unsaturated fatty acids to the cyclic ring of their cyclopropane derivatives as well as the

fabH; a gene which plays an important role in the first step of the elongation process of fatty acid biosynthesis were selected to evaluate whether acid adaptation could induce the transcription level of these genes. The results indicated that the c*fa* mRNA expression levels increased by approximately 12 fold after the cells were adapted in acidified MRS (pH 3, 25 °C, 1 h), relative to unmodified MRS (pH 6.4, 25 °C, 1 h). This increase in c*fa* mRNA coincides with the results in Table 2 showing that in the acid adapted cells the concentration of cis-11,12-methyleneoctadecanoic acid methyl ester (lactobacillic acid) increased significantly from 6.4% to 10.2% and that of cis-11-octadecenoic acid methyl ester (cis-vaccenic acid) decreased from 15.46% to 13.73% compared to the control cells. These results demonstrate the significant role of *cfa* in the process of acid adaptation. The induction of the *cfa* gene under low pH was also observed in previous work with *Lactococcus lactis* MG 1363 (Budin-Verneuil et al., 2005) and *L. plantarum* KR6 (Seme *et al*., 2015), although a different experimental set up was used. More specifically, the amount of *cfa* mRNA in *Lactococcus lactis* MG 1363 determined by northern blot analysis was higher when the cells were grown in acidified M17 medium with HCl (pH 5, 30 min) compared to cells grown in control M17 medium (pH 7, 30 min). Upregulation of the *cfa* gene was shown for *L. plantarum* KR6 cells exposed to acidified MRS with HCl (pH 4.5 and 2.5, 30 min) compared to control MRS (pH 7, 30 min).

The results also indicated that the levels of *fabH* mRNA increased by 6-fold after acid adaptation, although this difference was not statistically significant. Upregulation by 15-fold of *fabH* transcription has also been previously reported by Fernandez *et al*. (2008) in *L. bulgaricus* cells after exposure to MRS acidified with lactic acid to pH 3.8, and subsequently incubated in MRS (pH 4.9 with lactic acid, 40 min). Upregulation of *fabH* might increase the straight chain saturated fatty acids during acid adaptation in lactobacilli; however, the upregulation of *fabH* gene did not coincide with the saturated fatty acid profiles data between

acid adapted and control cells (Table 2) as no significant difference were observed. This discrepancy might be due to the overexpression of *cfa* gene which utilised newly synthesised unsaturated fatty acids for cyclopropane production as mentioned above. From these results, the straight chain saturated fatty acids were synthesized in *L. plantarum* NCIMB 8826 during acid adaptation but were converted to cyclopropane by *cfa* gene. Overall, findings from the present study indicated that the survival of *L. plantarum* NCIMB 8826 during storage in low pH fruit juices involved in the inhibition of the influx of hydrogen protons or antimicrobial compounds into the cells.

4 Conclusions

Considering the average shelf life of refrigerated fruit juices (~6 weeks) (Esteve and Frígola, 2007) and the concentration of viable cells required in probiotics (~10^7 CFU/ml) (Corcoran *et al*., 2007), the cell concentration of *L. plantarum* NCIMB 8826 in each fruit juice should be higher than ~10^7 CFU/ml after 6 weeks in order to exert its beneficial effects upon consumption. However, the above cell concentration was maintained only up to weeks 2 and 3 in pomegranate and lemon & lime juices, respectively. These results suggest that the modification of fatty acids particularly cyclopropane after acid adaptation was not sufficient to make these particular fruit juices suitable for the delivery of *L. plantarum* NCIMB 8826 in commercial products but this seems to be significant enough to improve the cell survival of the probiotic strains which are sensitive to the low pH of gastric juice during passage through the stomach as this process requires 4 to 5 h (Camilleri *et al*., 1989). However, the pomegranate and lemon & lime juices have the potential to be used as probiotic carriers as they provide a relatively mild environment for the cells, compared to the harsh environment of cranberry juice. To this end, new strategies that could prolong probiotic survival in these fruit juices, such as use of microencapsulation in polymeric matrices that can protect the cells

from acid, the use of other bacterial strains that are more robust to acidic environments, other ways of enhancement of acid resistance apart from exposure to low pH, or the use of other mild fruit juices that could enhance survival.

5 References

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Table 1. Target genes and primers used in RT-PCR analysis

Locus tag[a]	gene	Function	Primer(5'-3')	*E* value
lp_rRNA01	*16S rRNA*	16S ribosomal RNA	**Forward primer:** TCTGTAACTGACGCCTGAGGC **Reserve primer:** CTGTATCCATGTCCCCGAAG	1.98
lp_1696	*cfa*	Cyclopropane fatty acyl phospholipid synthase	**Forward primer:** AGCAGCGTCATTTGGAGGAA **Reserve primer:** GACCTGTTGTTTCGACCTGCT	2.23
lp_1671	*fabH2*	3-oxoacyl ACP synthase	**Forward primer:** GTGCGGGCTTTGTTTATGGG **Reserve primer:** CAGTGCCCAGTGGTCGTATT	1.97

[a]Designated gene loci for annotating the location on *L. plantarum* WCFS1 chromosome.

Table 2 Relative percentages of cellular fatty acids after incubation of *L. plantarum* cells in unmodified MRS (25 °C, pH 6.4, 1 h)[a] and acidified MRS (25 °C, pH 3, 1 h)[b].

Systematic Nomenclature	Lipid Numbers	Percentage of cellular fatty acids	
		Control cells[a]	Acid adapted cells[b]
Tetradecanoic Acid Methyl Ester	14:0	4.82±0.62	3.63±0.02
cis-9-Hexadecenoic Acid Methyl Ester	16:1w7c	8.10±1.31	6.33±0.10
Hexadecanoic Acid Methyl Ester	16:0	25.86±1.39	24.74±0.58
cis-9-Heptadecenoic Acid Methyl Ester	17:1w8c	0.83±0.06	0.71±0.01
cis-9-Octadecenoic Acid Methyl Ester	18:1w9c	11.40±1.32	11.06±0.11
cis-11-Octadecenoic Acid Methyl Ester	18:1w7c	15.46±2.43	13.73±0.01
Octadecanoic Acid Methyl Ester	18:0	1.24±0.51	1.26±0.14
cis-13-Nonadecenoic Acid Methyl Ester	19:1 w6c	25.65±0.21	27.32±1.06
cis-11,12-Methylene Octadecanoic Acid Methyl Ester	19:0 CYCLO w7c	6.39±0.41	10.22±0.69*

*indicates significant difference to control cells (p<0.05)

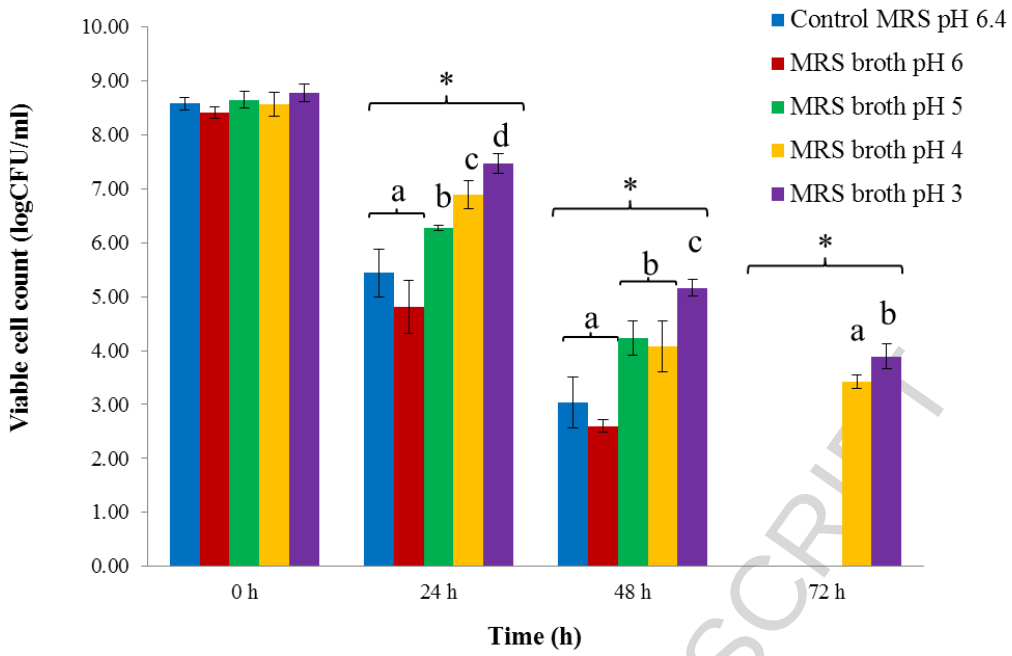

Figure 1. Survival of *L. plantarum* NCIMB 8826 during refrigerated storage in cranberry juice after pre-treatment in MRS acidified to different pH using citric acid. Results are expressed in mean±SD (n=3). Significant differences in cell viability between different pH within each group at a specific time point were determined by one-way Anova and Tukey's post-hoc tests. Asterisk indicates significant differences in cell viability between each pH determined by one-way Anova while ^{a}, ^{b}, ^{c}, ^{d} indicate significant difference in cell viability between each pH determined by Tukey's post-hoc tests.

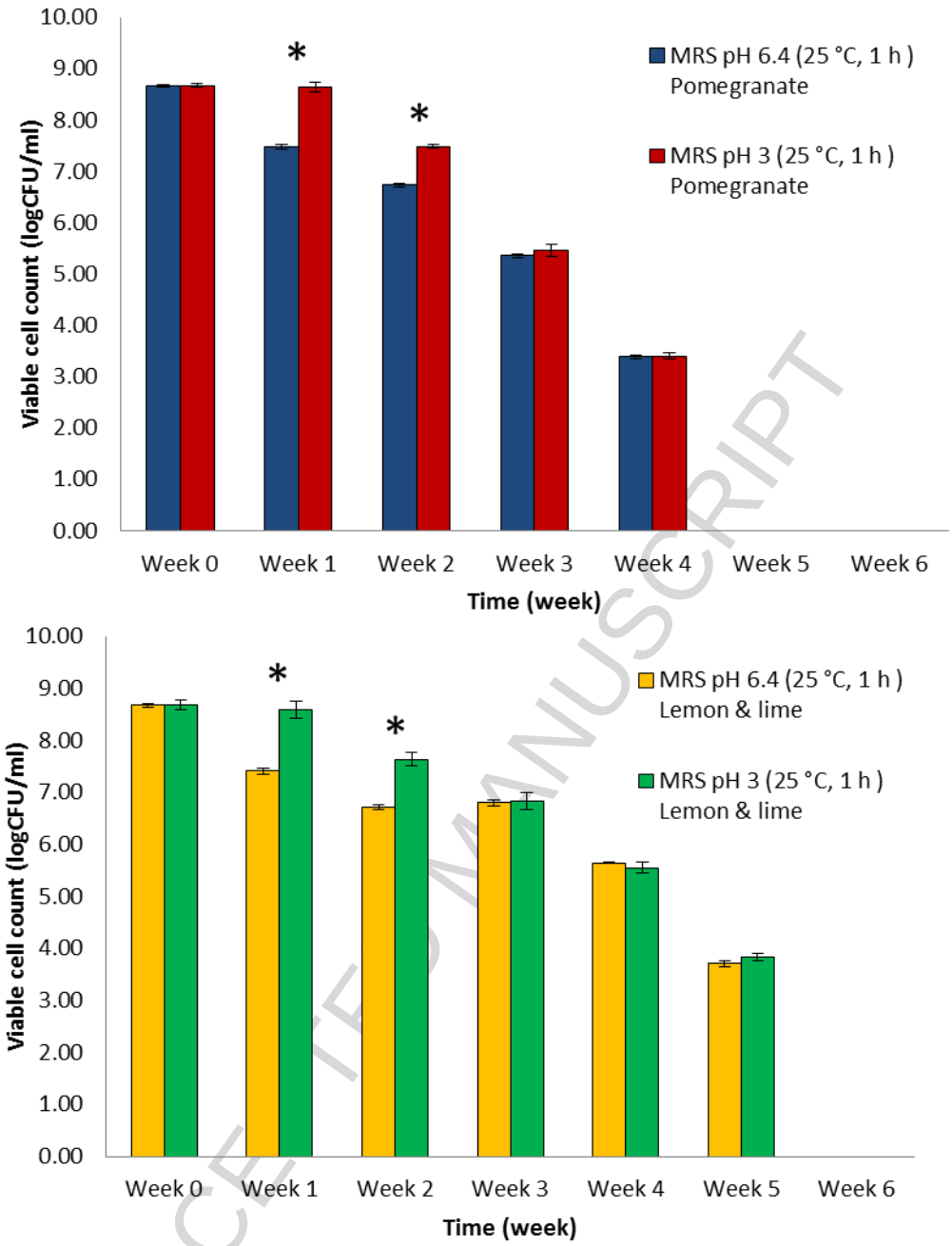

Figure 2. Survival of *L. plantarum* NCIMB 8826 during refrigerated storage in pomegranate (A), and lemon & lime (B) after pre-treatment in acidified MRS (pH 3, 25 °C, 1 h) and in unmodified MRS (pH 6.4, 25 °C, 1 h). Results are expressed in mean±SD (n=3). Significant differences in cell viability between acidified cells (pH 3) and control cells (pH 6.4) at specific time point were determined by paired t-test. Asterisk indicates significant differences.

Highlights

- Citric acid adaptation improved the survival of *Lactobacillus plantarum* NCMIB 8826 in highly acidic fruit juices (pH 2.7 to 3.5).
- Cranberry juice was more toxic towards *L. plantarum* NCMIB 8826 than pomegranate and lemon & lime juices
- Increase in cyclopropane fatty acid due to adaptation in citric acid improved survival of *L. plantarum* NCIMB 8826 during storage in all three acidic fruit juices
- The expression of cyclopropane synthase (*cfa*) was increased about 12-fold due to citric acid adaptation.